Anal. Chem. **2001**, *73*, 2578–2586

Phosphoprotein Isotope-Coded Affinity Tag Approach for Isolating and Quantitating Phosphopeptides in Proteome-Wide Analyses

Michael B. Goshe, Thomas P. Conrads, Ellen A. Panisko, Nicolas H. Angell, Timothy D. Veenstra, and Richard D. Smith*

Environmental and Molecular Sciences Laboratory, Pacific Northwest National Laboratory, P.O. Box 999, MSIN: K8-98, Richland, Washington 99352

A method has been developed that utilizes phosphoprotein isotope-coded affinity tags (PhIAT) that combines stable isotope and biotin labeling to enrich and quantitatively measure differences in the O-phosphorylation states of proteins. The PhIAT labeling approach involves hydroxide ion-mediated β-elimination of the *O*-phosphate moiety and the addition of 1,2-ethanedithiol containing either four alkyl hydrogens (EDT-D_0) or four alkyl deuteriums (EDT-D_4) followed by biotinylation of the EDT-D_0/D_4 moiety using (+)-biotinyl-iodoacetamidyl-3,6-dioxaoctanediamine. The PhIAT reagent, which contains the nucleophilic sulfhydryl and isotopic label covalently linked to a biotin moiety, was synthesized and has the potential utility to reduce the O-phosphorylation derivatization into a one-step process. The PhIAT labeling approach was initially demonstrated using the model phosphoprotein β-casein. After proteolytic digestion, the PhIAT-labeled peptides were affinity isolated using immobilized avidin and analyzed using capillary reversed-phase liquid chromatography–mass spectrometry. PhIAT-labeled β-casein peptides corresponding to peptides containing known sites of O-phosphorylation were isolated and identified. The PhIAT labeling method was also applied to a yeast protein extract. The PhIAT labeling technique provides a reliable method for making quantitative measurements of differences in the O-phosphorylation state of proteins.

The availability of complete genome sequences is moving biological research to an era where cellular systems are analyzed as a whole rather than analyzing the individual components. While genome sequences and global gene expression measurements at the mRNA level opens the door to important biological advances,[1,2] much of the understanding of cellular systems and the roles of the constituents will arise from proteomics.[3,4] Proteomics, the analysis of the entire complement of proteins expressed by a cell, tissue, or organism, provides the most informative characterization of the cell system since proteins are the primary players responsible for nearly all cellular processes. A key aspect to successful proteomic measurements is the ability to precisely measure protein abundance changes in a high-throughput manner. This allows the effects of many "perturbations" upon, or changes to, a cell type, tissue, or organ to be determined in a rapid fashion.[5] An inherent goal of proteomic studies is to provide a greater understanding of the function of proteins within a global, cellular context, along with the more conventionally delineated molecular function. The study of proteins at the level of cellular systems will provide a stronger basis for understanding complex biological pathways and the nature of diseases and will permit a starting point for developing predictive capabilities in modeling the effects of cellular insults. The global understanding of cellular systems revealed by proteomic investigations will create new avenues of research unlikely to originate from the past paradigm of "single" protein characterization methodologies.

Aebersold and co-workers have recently reported an approach for high-throughput proteome quantitation that employs isotope-coded affinity tags (ICAT).[6] This method involves the affinity isolation of cysteine-containing polypeptides (Cys-polypeptides) by modifying the proteins with a Cys-specific reagent that contains a linker arm connecting a thiol-reactive group to a biotin moiety. The ICAT reagent is used in both a "light" and a "heavy" isotopic version where eight hydrogen atoms in the linker arm of the light reagent (D_0) have been substituted by eight deuterium atoms in the heavy reagent (D_8). Derivatization of two distinct proteomes with the light and heavy versions of the ICAT reagent provides a basis for protein quantitation. In addition, the presence of a cysteinyl residue within the labeled peptides provides an additional constraint that serves to increase the confidence of peptide identification.

While the ICAT approach shows great promise for determining relative protein abundances, delineation of protein function solely from abundance changes will be limited since numerous vital activities of proteins are modulated by posttranslational modifications that may not be reflected by changes in protein abundance.

(1) Wasinger, V. C.; Cordwell, S. J.; Cerpa-Poljak, A.; Yan, J. X.; Gooley, A. A.; Wilkins, M. R.; Duncan, M. W.; Harris, R.; Williams, K. L.; Humphery-Smith, I. *Electrophoresis* **1995**, *16*, 1090–4.

(2) Patterson, S. D. *Curr. Opin. Biotechnol.* **2000**, *11*, 413–8.

(3) Schena, M.; Shalon, D.; Davis, R. W.; Brown, P. O. *Science* **1995**, *270*, 467–70.

(4) Khodursky, A. B.; Peter, B. J.; Cozzarelli, N. R.; Botstein, D.; Brown, P. O.; Yanofsky, C. *Proc. Natl. Acad. Sci. U.S.A.* **2000**, *97*, 12170–5.

(5) Gygi, S. P.; Corthals, G. L.; Zhang, Y.; Rochon, Y.; Aebersold, R. *Proc. Natl. Acad. Sci. U.S.A.* **2000**, *97*, 9390–5.

(6) Gygi, S. P.; Rist, B.; Gerber, S. A.; Turecek, F.; Gelb, M. H.; Aebersold, R. *Nat. Biotechnol.* **1999**, *17*, 994–9.

1/ac010081x CCC: $20.00
Published on Web 04/28/2001

One of the most important posttranslational protein modifications used to modulate protein activity and propagate signals within cellular pathways and networks is phosphorylation.[7-9] Studies estimate that as many as one-third of all cellular proteins derived from mammalian cells are phosphorylated.[7] Cellular processes ranging from protein kinase activation, cell cycle progression, cellular differentiation and transformation, development, and peptide hormone response, to adaptation are all regulated by changes in the state of protein phosphorylation. While regulation can occur at the level of protein synthesis along with concomitant proportional changes in protein phosphorylation, there are also examples of regulating protein function by phosphorylation without altering protein abundance.[10] Therefore, the ability to broadly identify changes in the phosphorylation state of a protein may lead to discoveries related to protein activity—regardless of whether that protein is differentially expressed—and improve our understanding of cellular systems.

The predominant method used to study changes in protein phosphorylation is radiolabeling proteins with ^{32}P inorganic phosphate ($^{32}P_i$). To measure differences in relative abundances of phosphorylation, ^{32}P-labeled proteomes are analyzed by 2-D polyacrylamide gel electrophoresis (2-D PAGE) and the relative spot intensities are compared.[11,12] The use of $^{32}P_i$ to label proteins does not lend itself to high-throughput proteome-wide analysis due to the problems with handling radioactive compounds and the associated contamination of instrumentation. It would be valuable to develop alternative methods posing less of a health risk than the use of $^{32}P_i$, yet still provide the capability to effectively identify phosphorylated proteins and quantitate the extent of phosphorylation. Two methods using heavy isotope metabolic labeling of proteins[13] or derivatization of phosphopeptides with isotopically distinct reagents after protein extraction from the cell[14,15] have been proposed. While both of these strategies are potentially effective at quantitating phosphopeptides, only one of these has the ability to isolate phosphopeptides from complex mixtures.[15] Immunoaffinity and metal-affinity columns designed for enrichment of phosphopeptides often result in the isolation of many nonphosphorylated peptides through nonspecific interactions. These nonspecifically bound components complicate the downstream analysis by introducing uncertainty about the nature of the sample. The ability to prepare proteome samples that are more highly enriched in phosphorylated peptides would greatly simplify the identification of these species. In addition, due to the low abundance of typical phosphopeptides and the finite dynamic range of present analytical technologies, the detection of phosphopeptides would be enhanced if nonphosphorylated peptides were not present in the sample to be analyzed.

Although greater emphasis is being directed toward more global analysis of cellular systems, methodologies still do not exist for reliable, high-throughput analysis of proteome-wide changes in the phosphorylation of proteins. In an effort to address this issue, a novel strategy has been developed to identify and quantitate the extent of protein phosphorylation using a phosphoprotein isotope-coded affinity tag (PhIAT). The PhIAT methodology shares the virtues of the ICAT approach to proteomic analysis: a stable isotope label possessing a biotin tag that permits proteome-wide purification and quantitation of peptides containing specific types of residues.

EXPERIMENTAL SECTION

Materials. The materials used in all experiments were obtained from commercially available sources and used without further purification unless otherwise noted. β-Casein (from bovine milk, minimum 90% purity) was obtained from Sigma. The 1,2-ethanedithiol (EDT, $HSCH_2CH_2SH$, >98%) was purchased from Fluka. The ethane-d_4-1,2-dithiol ($HSCD_2CD_2SH$, 99 atom % D) was purchased from C/D/N Isotopes (Pointe-Claire, PQ, Canada). (+)-Biotinyl-iodoacetamidyl-3,6-dioxaoctanediamine (iodoacetyl-PEO-biotin) and tris(2-carboxyethyl)phosphine hydrochloride (TCEP-HCl) were obtained from Pierce (Rockford, IL). Acetonitrile (HPLC grade) and glacial acetic acid (ACS reagent grade) were purchased from Aldrich (Milwaukee, WI), and trifluoroacetic acid (TFA) (HPLC grade) was purchased from Sigma (St. Louis, MO). Water was purified using a Barnstead Nanopure Infinity water purification system (Dubuque, IA).

β-Casein Sample Preparation. A stock solution of 10 mg/mL β-casein was prepared by dissolving lyophilized protein into 0.1 M NH_4HCO_3, pH 8.2, containing 6 M guanidine hydrochloride (GdnHCl). Aliquots, varying from 0.1 to 1 mg, were removed, lyophilized, and stored at −20 °C until PhIAT labeling was performed.

Yeast Strain and Growth Conditions. *Saccharomyces cerevisiae* strain S288C (1) (*MAT*α, *SUC2, mal, mel, gal2, CUP1, flo1, flo8-1*) was grown in rich media (1% yeast extract, 2% Bactopeptone) with glucose (2%, final concentration) as the carbon source. Cultures were inoculated at OD_{600} < 0.1, incubated at 30 °C with shaking, and harvested at $OD_{600} \cong 1.0$. Cell pellets were collected by centrifugation at 2000*g* and stored at −80 °C until needed.

Preparation of Yeast Whole Cell Lysate. The yeast cell pellet was resuspended in 500 μL of 0.1 M NH_4HCO_3, pH 8.2, and one-third volume of 425−600-μm acid-washed glass beads (Sigma) was added. Cells were broken with a Mini Bead-Beater (Biospec Products, Bartlesville, OK) operating at 6000 rpm for 1 min followed by a 1-min incubation on ice. This procedure was repeated twice, and the extract was transferred to a new tube. The glass beads were rinsed with 200 μL of 0.1 M NH_4HCO_3, pH 8.2, and the wash was added to the reserved extract. Cell debris was removed from the extract by centrifugation for 15 min at 12000*g*. The supernatant was collected and protein concentration was determined by the bicinchoninic acid (BCA) assay[16] from Pierce. The extract was denatured by addition of 6 M GdnHCl

(7) Pawson, T.; Scott, J. D. *Science* **1997**, *278*, 2075−80.
(8) Cohen, P. *Nature* **1982**, *296*, 613−20.
(9) Cohen, P. *Trends Biochem. Sci.* **1992**, *17*, 408−13.
(10) Huang, C.; Ma, W. Y.; Young, M. R.; Colburn, N.; Dong, Z. *Proc. Natl. Acad. Sci. U.S.A.* **1998**, *95*, 156−61.
(11) van der Geer, P.; Hunter, T. *Electrophoresis* **1994**, *15*, 544−54.
(12) Mason, G. G.; Murray, R. Z.; Pappin, D.; Rivett, A. J. *FEBS Lett.* **1998**, *430*, 269−74.
(13) Oda, Y.; Huang, K.; Cross, F. R.; Cowburn, D.; Chait, B. T. *Proc. Natl. Acad. Sci. U.S.A.* **1999**, *96*, 6591−6.
(14) Weckwerth, W.; Willmitzer, L.; Fiehn, O. *Rapid Commun. Mass Spectrom.* **2000**, *14*, 1677−81.
(15) Zhou, H.; Watts, J.; Aebersold, R. *The 48th ASMS Conference on Mass Spectrometry and Allied Topics*, Long Beach, CA, June 11−15, 2000.
(16) Smith, P. K.; Krohn, R. I.; Hermanson, G. T.; Mallia, A. K.; Gartner, F. H.; Provenzano, M. D.; Fujimoto, E. K.; Goeke, N. M.; Olson, B. J.; Klenk, D. C. *Anal. Biochem.* **1985**, *150*, 76−85.

and boiled for 5 min. After cooling to room temperature, a 30 molar excess of TCEP-HCl to protein was added to reduce disulfide bonds. The sample was incubated at 37 °C for 45 min. A 10 molar excess of iodoacetate over cysteinyl residues was added, and the sample was incubated with stirring at ambient temperature in the dark for 90 min. A PD-10 column (Pierce) equilibrated with 0.1 M NH_4HCO_3, pH 8.2, was used to desalt the sample. The protein concentration of the pooled fractions was determined by BCA assay.

β-Elimination and PhIAT Labeling. The protein sample was divided into two equal aliquots and labeled with EDT-D_0 ($HSCH_2CH_2SH$) or EDT-D_4 ($HSCD_2CD_2SH$), respectively. The reaction conditions for β-elimination and EDT labeling are based upon previously published methods describing the β-elimination of phosphate with subsequent Michael addition of various sulfhydryl nucleophiles.[17-19] The reaction conditions, however, were modified to facilitate differential labeling of a complex protein mixture or proteome as described below.

The β-elimination/EDT reaction mixture consisted of the following components: 150 μL of H_2O, 185 μL of dimethyl sulfoxide, 35 μL of ethanol, 35 μL of acetonitrile, 5 μL of 250 mM EDTA (pH 8.0), 45 μL of 5 M NaOH, and 11 μL of either EDT-D_0 or EDT-D_4. All solvents (except for EDT) were degassed with N_2 for 5 min before and after preparation of the reaction mixture just prior to EDT addition. After EDT addition, 200 μL of the β-elimination/EDT reaction mixture was added to the lyophilized protein sample (a maximum of 1 mg of cysteinyl blocked protein per 200 μL of the β-elimination/EDT reaction mixture). The samples were incubated for 1 h at 55 °C under a N_2 atmosphere, cooled to room temperature, and the reaction quenched by neutralizing with acetic acid.

After the reaction was quenched, the protein samples labeled with EDT-D_0 or EDT-D_4 were combined and desalted using a PD-10 column (Pierce) equilibrated with 0.1 M NH_4HCO_3, pH 8.2. The protein and sulfhydryl content were quantified using the BCA assay and 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) assay,[20] respectively. At this stage of PhIAT labeling, the EDT-D_0/D_4-labeled protein was either biotinlyated or lyophilized and stored at −80 °C until biotinlyation was later performed.

Biotinylation of EDT-Labeled Protein. The lyophilized EDT-labeled proteins were resuspended in 1 mL of 0.1 M NH_4HCO_3, pH 8.2, denatured by adding GdnHCl to 6 M, and reduced with TCEP-HCl as described above. The EDT-labeled residues were biotinylated by adding a 5 molar excess of iodoacetyl-PEO-biotin (sulfhydryl content determined by DTNB assay or estimated based on the concentration of protein present as determined by BCA assay). The biotinylation reaction was allowed to proceed with constant stirring in the dark for 90 min at ambient temperature. The EDT-acetyl-PEO-biotin-labeled proteins (PhIAT-D_0/D_4-labeled proteins) were desalted into 0.1 M NH_4HCO_3, pH 8.2, and digested overnight at 37 °C with sequence grade modified trypsin (Promega, Madison, WI) using a 1:50 (w:w) trypsin-to-protein ratio. Tryptic activity was quenched by boiling the sample followed by the addition of phenylmethanesulfonyl fluoride to a final concentration of 1 mM.

Affinity Purification of PhIAT Peptides. The PhIAT-labeled peptides were purified by affinity chromatography using ImmunoPure immobilized monomeric avidin from Pierce as follows. Between 1.2 and 1.5 mL of avidin slurry was packed in a glass pasture pipet containing a glass wool plug. Prior to sample loading, irreversible biotin binding sites were blocked as per manufacturer's instructions. The sample containing the PhIAT-labeled peptides was added to the column and permitted to incubate for 30 min at ambient temperature. The column was washed with 15 mL of 2× phosphate-buffered saline (PBS) (0.2 M sodium phosphate, 0.3 M NaCl, pH 7.2), 15 mL of 1× PBS (0.1 M sodium phosphate, 0.15 M NaCl, pH 7.2), and 15 mL of 0.1 M NH_4HCO_3, pH 8.2. The PhIAT-labeled peptides were eluted using 2 mL of 30% acetonitrile/0.4% TFA. The recovery of PhIAT-labeled peptides was quantified using the BCA assay. The PhIAT-labeled peptides were lyophilized and stored at −20 °C.

Synthesis of the PhIAT Reagent. All solvents (except for EDT) were degassed with N_2 for 5 min and then, after these solvents were combined in the appropriate proportions, the resulting solutions were degassed with N_2 for several more minutes. A solution of 0.1 M TCEP-HCl in 0.2 M Tris, pH 8.4, was prepared and the pH of this reducing solution adjusted to 8.3 using 5 M NaOH. The PhIAT reaction solution was made by combining the following reagents in a glass sample vial containing a stirring bar: 250 μL of 0.1 M TCEP-HCl in 0.2 M Tris, 25 μL of 250 mM EDTA, pH 8.0, 225 μL of ethanol, and 400 μL of 2% EDT-D_0 in ethanol. After mixing, 5 mg of iodoacetyl-PEO-biotin was added, followed by an additional 250 μL of the reducing solution. Once the iodoacetyl-PEO-biotin was thoroughly dissolved, the headspace in the reaction vessel was exposed to N_2 for several seconds and sealed with a silicon/Teflon septum. The reaction was permitted to proceed with constant stirring in the dark for 2.5 h at ambient temperature and was quenched by neutralizing with 80 μL of 10% acetic acid. To completely quench the reaction, the apparent pH of the reaction solution should be 6. Upon neutralization, 600 μL of H_2O was added prior to ether extraction.

Excess EDT and any potential disulfide-linked EDT byproducts were removed by extracting twice with equal volumes of water-saturated diethyl ether containing 30% ethanol. Residual diethyl ether was removed over a stream of N_2. The PhIAT reagent (EDT-D_0-acetyl-PEO-biotin) was isolated by affinity chromatography using immobilized avidin. The eluant (PhIAT reagent) was lyophilized and stored at −20 °C.

Capillary LC−MS Analysis. The HPLC system consisted of a Gilson model 321 pump and 235P autoinjector, both controlled via Unipoint System software (Gilson Inc., Middleton, WI). A reversed-phase capillary HPLC column was manufactured in-house by slurry packing 3-μm Jupiter C_{18} stationary phase (Phenomenex, Torrence, CA) into a 65 cm, 360 μm o.d. × 150 μm i.d., capillary (Polymicro Technologies Inc., Phoenix, AZ) incorporating a 2-μm retaining mesh in a HPLC union (Valco Instruments Co., Houston, TX). The mobile phase consisted of 0.2% acetic acid and 0.05% TFA in water (A) and 0.1% TFA in 90% acetonitrile/10% water (B) and was degassed on-line using a vacuum degasser (Jones Chromatography Inc., Lakewood, CO). The HPLC pump flow, 300 μL/min, was split through a capillary microtee assembly (Up-

(17) Meyer, H. E.; Hoffmann-Posorske, E.; Korte, H.; Heilmeyer, L. M., Jr. *FEBS Lett.* **1986**, *204*, 61−6.
(18) Mega, T.; Hamazume, Y.; Hong, Y. M.; Ikenaka, T.; Nong, Y. M. *J. Biochem. (Tokyo)* **1986**, *100*, 1109−16.
(19) Fadden, P.; Haystead, T. A. *Anal. Biochem.* **1995**, *225*, 81−8.
(20) Ellman, G. L. *Arch. Biochem. Biophys.* **1959**, *82*, 70.

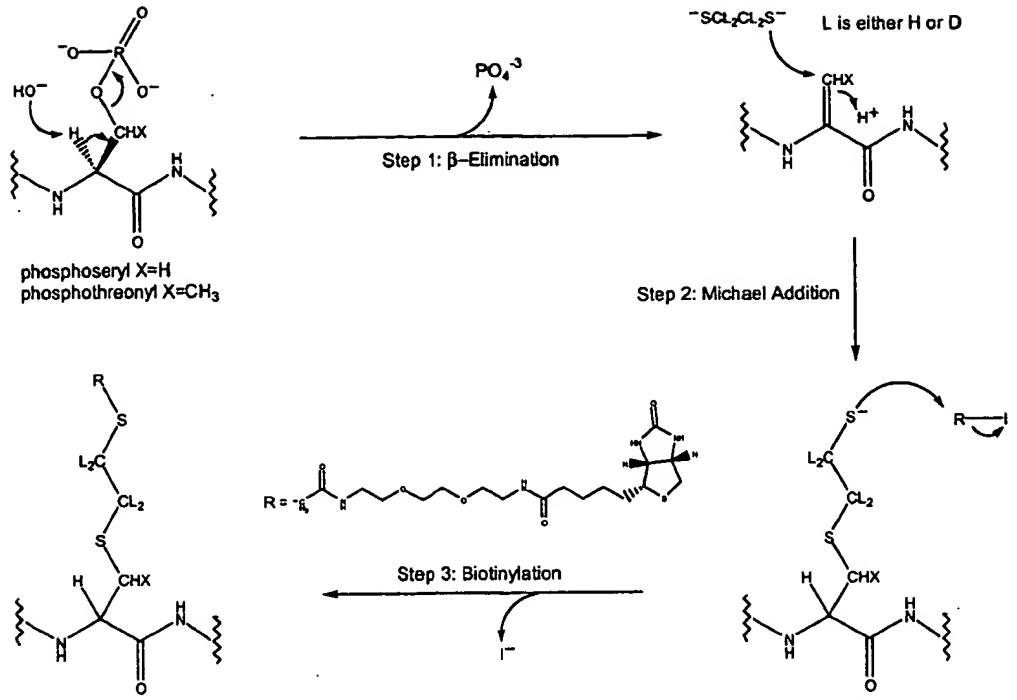

Figure 1. Phosphoprotein isotope-coded affinity tag labeling approach. Proteins containing phosphoseryl (X = H) or phosphothreonyl (X = CH_3) residues are isotopically labeled and biotinylated. After proteolytic digestion, biotinylated peptides are isolated from nonphosphorylated peptides via avidin affinity chromatography. The ability to quantitate the extent of phosphorylation between two identical peptides extracted from different sources is based on the use of a light (L = H, EDT-D_0) and heavy (L = D, EDT-D_4) isotopic versions of 1,2-ethanedithiol.

church Scientific, Oak Harbor, WA) before the autoinjector to establish a measured flow through the column of 1.5 μL/min. After a sample volume of 10 μL was injected onto the reversed-phase capillary HPLC column, the mobile phase was held at 100% A for 10 min followed by a linear gradient to 100% B over 90 min and then held at 100% B for 20 min. The column was then reequilibrated with 100% A prior to the next injection.

LC–MS analysis (scan range 400–2000 Da) was performed using an ion trap mass spectrometer (LCQ, Finnigan MAT, San Jose, CA) with electrospray ionization (ESI). The LC capillary was coupled to the mass spectrometer using an in-house-manufactured ESI interface. The ESI conditions were as follows: spray voltage, 3.0 kV; capillary temperature, 150 °C; capillary voltage, 5 V; tube lens offset, 0 V; no sheath gas or makeup liquid was used. Additional LC–MS analysis (range 600–4000 Da) was performed using a hybrid quadrupole-TOF mass spectrometer (API QStar Pulsar, PE Sciex Instruments, Toronto, Canada) equipped with a PE Sciex Instruments MicroIonSpray ESI interface in the TOF-only mode. ESI conditions were as follows: spray voltage, 5.5 kV; nebulizing gas, 8; curtain gas, 35; no makeup liquid was used.

RESULTS AND DISCUSSION

The reversible phosphorylation of proteins plays a major role in many vital cellular processes. Because phosphorylation is dynamic and the sites of phosphorylation cannot be predicted by an organism's genome, proteomic measurements are needed to identify sites of and changes in the phosphorylation state of proteins. Since a high percentage of proteins in higher eukaryotic organisms are predicted to be phosphorylated, methods to identify and quantitate phosphorylation sites should ideally be high-throughput, enabling proteome-wide changes in these sites to be identified in a single experiment. Unfortunately, current methodologies have proven inadequate partially due to the lack of a phosphorylation site-specific reagent and the difficulties associated with confidently identify phosphorylated peptides in a complex mixture of peptides.

To address this need we have developed a method that utilizes a "phosphoprotein isotope-coded affinity tag" to differentially label phosphoseryl (pSer) and phosphothreonyl (pThr) residues with a stable isotopic and biotinylated tag that permits the enrichment and quantification of phosphopeptides from a proteome. The central theme to the PhIAT strategy is that the phosophoprotein pool is enriched in a manner that enables a quantitative measurement of phosphorylation to be made between the two distinct protein samples by comparing the extent of isotopic enrichment.

The PhIAT labeling procedure is shown in Figure 1. Phosphoproteins are chemically modified by removing the phosphate group from pSer and pThr residues (step 1) via hydroxide ion-mediated β-elimination, resulting in dehydroalanyl and β-methyldehydroalanyl residues, respectively. The formation of an α,β-unsaturated double bond produces an electrophilic center at the β-position of these formerly phosphorylated residues. This reactive β-carbon is susceptible to nucleophilic attack and is modified by EDT via Michael addition to a form a covalent thioether linkage (step 2). The addition of EDT converts the dehydroalanyl and β-methyldehydroalanyl residues into residues that contain nucleophilic sulfhydryls. Stable isotopic labeling is achieved by using either the light (EDT-D_0) or the heavy (EDT-D_4) isotope of EDT. The sulfhydryls present on the EDT-labeled proteins are biotinylated (step 3) to generate the PhIAT-labeled protein via SN2

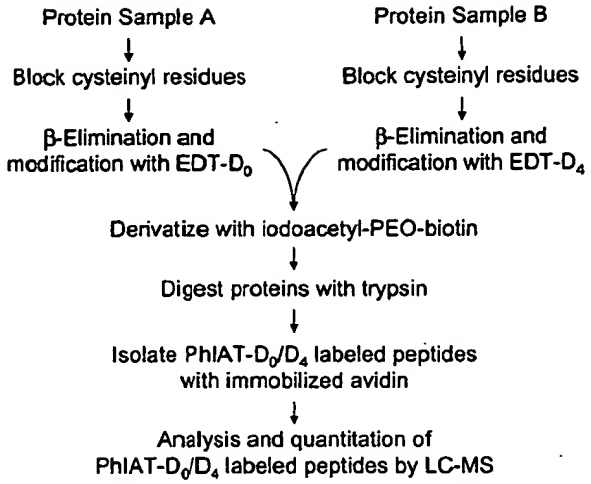

Figure 2. Analytical scheme for the relative quantitation and isolation of phosphopeptides from two different samples using the PhIAT labeling approach.

displacement of iodide from iodoacetyl-PEO-biotin. The PhIAT-labeled proteins are digested with trypsin and immobilized avidin is used to isolate the PhIAT-labeled peptides prior to LC-MS analysis.

PhIAT Labeling of β-Casein. To demonstrate the ability of the PhIAT method to isolate and quantitate phosphopeptides, two equivalent samples of β-casein were modified as outlined in Figure 2. Each sample was treated with either EDT-D_0 or EDT-D_4 and then combined prior to derivatization with iodoacetyl-PEO-biotin. After biotinylation and digestion with trypsin, the PhIAT-labeled peptides were isolated using immobilized avidin chromatography and then analyzed by LC–MS. The PhIAT-labeled peptides are identified as pairs that differ in mass according to the number of EDT-D_0 and EDT-D_4 labels. For the two peptides containing a single pSer residue, pairs of peaks were detected with the mass differences equivalent to the mass difference between the light (PhIAT-D_0) and heavy (PhIAT-D_4) isotopic versions of the PhIAT label. The mass spectra of the phosphorylated peptide FQS^{P}EEQQQTEDELQDK in which the pSer residue has been modified with EDT (D_0 or D_4) and iodoacetyl-PEO-biotin (PhIAT labeled) is shown in Figure 3. On the basis of the higher resolution spectrum in Figure 3B, the 2.01 m/z difference between the $[M + 2H]^{2+}$ ions at m/z 1236.96 and 1238.97, corresponds to a 4.02 Da mass difference, which is the expected mass difference resulting from PhIAT labeling one sample with EDT-D_0 and the other with EDT-D_4. The efficiency of the 1:1 labeling, in which equivalent samples were labeled separately and pooled as outlined in Figure 2, is clearly evident by the equal intensity of the PhIAT-D_0- and PhIAT-D_4-labeled peptides. Table 1 lists the m/z values for the $[M + 2H]^{2+}$ ions of the PhIAT-labeled peptides for β-casein that were identified by LC–MS. The observed values are in good agreement with the calculated masses of the peptides.

The N-terminal tryptic peptide of β-casein (RELEELNVPGEIVES^{P}LS^{P}S^{P}S^{P}EESITR) contains four pSer residues in close proximity to one another. The ability to PhIAT label this portion of β-casein was examined by separately characterizing the extent of EDT labeling and biotinylation. Prior to the biotinylation step, an aliquot of EDT-D_0/D_4-labeled protein was digested with trypsin and analyzed by LC–MS. The observed $[M + 2H]^{2+}$ peak doublet

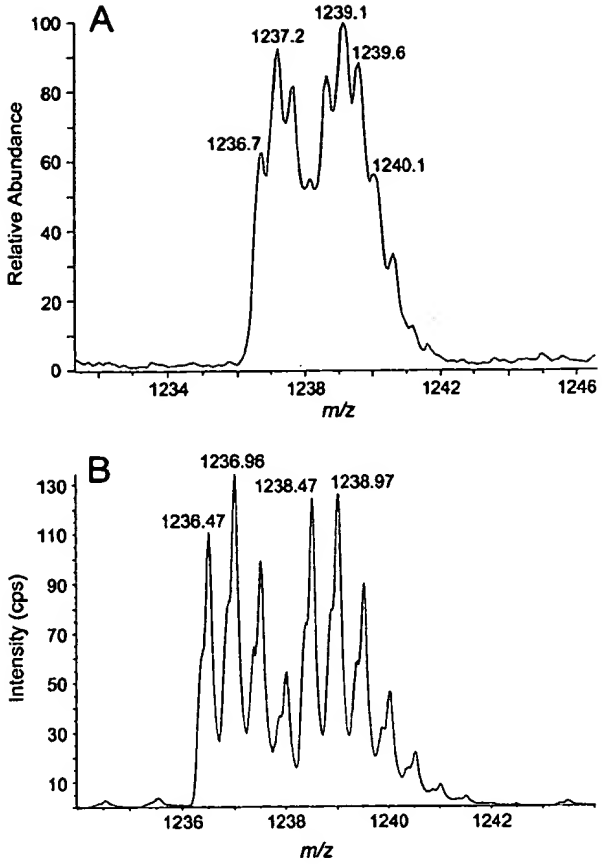

Figure 3. Mass spectra of PhIAT-D_0/D_4-labeled β-casein peptides. The enriched mixture of biotinylated phosphopeptides was analyzed by capillary reversed-phase liquid chromatography coupled directly on-line to (A) a Finnigan MAT LCQ ion trap mass spectrometer and (B) a PE Sciex API QStar Pulsar hybrid quadrupole-TOF mass spectrometer using approximately 1.0 and 0.5 μg/μL overall sample concentration per 10-μL injection, respectively. The $[M + 2H]^{2+}$ ion pair corresponds to the mass of the PhIAT-D_0/D_4-derivatized β-casein phosphopeptide FQS*EEQQQTEDELQDK, where S* has the modified side chain $-CH_2-SCL_2CL_2S$-acetyl-PEO-biotin and L is either H (PhIAT-D_0 label) or D (PhIAT-D_4 label).

at m/z 1552.4 and 1560.4, corresponding to a 16.0 Da mass difference between the EDT-labeled peptides, indicates that all four pSer were converted into the EDT-labeled derivative. The 2 Da difference between the calculated and observed m/z values may be the result of intramolecular disulfide bond formation between adjacent sulfhydryl groups. To prevent this type of disulfide bond formation during PhIAT labeling, the sample is reduced prior to biotinylation in order to remove any potential EDT disulfide bonds that may form during sample processing. Although an extreme case of phosphorylation, the ability to label a five-residue segment of a peptide containing up to four pSer residues (three of which are consecutive) demonstrates that the β-elimination/EDT labeling conditions are sufficient to begin examining protein phosphorylation sites that may be in close in proximity with one another in the primary sequence.

Biotinylated versions of the EDT-D_0/D_4-labeled N-terminal tryptic peptide were also detected by LC–MS as shown in Table 1. The doubly charged peptide pairs at m/z 1761.9 and 1769.5 corresponds to the N-terminal tryptic peptide in which all four phosphoseryl residues were labeled with EDT (D_0 or D_4) and one

Table 1. Detected[a] Phosphopeptides of β-Casein Using the PhIAT Labeling Approach

residues[b]	peptide sequence	PhIAT-D_0 labeled $[M + 2H]^{2+}$ calcd. *m/z*	PhIAT-D_0 labeled $[M + 2H]^{2+}$ obsd. *m/z*	PhIAT-D_4 labeled $[M + 2H]^{2+}$ calcd. *m/z*	PhIAT-D_4 labeled $[M + 2H]^{2+}$ obsd. *m/z*	no. of labels[c] PhIAT	no. of labels[c] EDT
45–63	IEKFQS*EEQQQTEDELQDK[d]	1422.6	1422.1	1424.6	1424.1	1	0
48–63	FQS*EEQQQTEDELQDK[d]	1237.4	1237.1	1239.4	1239.1	1	0
16–40	RELEELNVPGEIVES†LS†S†S†EESITR[e]	1762.2	1761.9	1770.1	1769.5	1	3
16–40	RELEELNVPGEIVES†LS†S†S†EESITR[e]	1969.4	1968.0	1977.4	1976.0	2	2

residues[b]	peptide sequence	EDT-D_0 labeled $[M + 2H]^{2+}$ calcd, *m/z*	EDT-D_0 labeled $[M + 2H]^{2+}$ obsd, *m/z*	EDT-D_4 labeled $[M + 2H]^{2+}$ calcd, *m/z*	EDT-D_4 labeled $[M + 2H]^{2+}$ obsd, *m/z*	no. of labels PhIAT	no. of labels EDT
16–40	RELEELNVPGEIVES†LS†S†S†EESITR[f]	1554.9	1552.4	1562.9	1560.4	0	4

[a] The observed *m/z* values were obtained from LC–MS data acquired with a Finnigan MAT LCQ ion trap mass spectrometer. The calculated *m/z* values are based on the average molecular weight of the modified peptides. [b] The β-casein sequence and the sites of phosphorylation were obtained from the Swiss-Prot entry P02666 (casb_bovin) and references contained therein. [c] The number and type of label were determined based on the observed and predicted masses of the modified peptides. [d] S* denotes pSer residues that have been labeled with PhIAT-D_0/D_4. [e] S† denotes pSer residues that have been labeled with either PhIAT-D_0/D_4 or EDT-D_0/D_4. [f] S† denotes pSer residues that have been labeled with EDT-D_0/D_4.

of these sites was also modified with the biotinylated tag. The doubly charged peptide pair at *m/z* 1968.0 and 1976.0 corresponds to the peptide in which two of the EDT-modified sites were also modified with the biotinylated tag. Although PhIAT-labeled peptides containing three and four biotin moieties were not confirmed due to the limited *m/z* range of the Finnigan MAT LCQ ion trap mass spectrometer used in this work, the attachment of one biotin is sufficient to isolate the peptide by avidin affinity. Since the isotopic label is contained within the EDT moiety, quantitation can still be performed.

PhIAT Labeling of a Yeast Proteome. Having demonstrated the utility of the PhIAT method to isolate phosphopeptides from β-casein, the method was applied to the whole-cell lysate from *S. cerevisiae* according to the steps outlined in Figure 2. The LC–MS analysis of the enriched PhIAT-labeled sample produced many isotopic doublets indicative of successful labeling of putative phosphoproteins. A few of the better PhIAT-D_0/D_4 pairs are shown in Figure 4. Each $[M + H]^+$ ion pair displays two essential features characteristic of the PhIAT labeling approach. The first is that each isotopic distribution differs by 4 Da, in accordance with the labeling performed with the D_0 and D_4 versions of the PhIAT label. The second is the 1:1 ratio displayed by each doublet, an indicator of the stoichiometric labeling of identical yeast protein samples with PhIAT-D_0 or PhIAT-D_4. Although no attempt was made to identify the proteins corresponding to the PhIAT-labeled phosphopeptides, it does demonstrate the ability to isolate and enrich for phosphopeptides from yeast. Experiments using LC–MS/MS with subsequent database searching of the product ions are currently being conducted and will provide the information necessary for protein identification and residue assignment of the PhIAT label.

The PhIAT labeling of the yeast protein extract displayed in Figure 4 was performed using 1 mg of protein, but only a percentage of this is phosphoprotein. Preliminary PhIAT labeling studies with β-casein indicated an overall sample loss of nearly 40% as determined by the BCA assay. The steps for denaturing, reduction, EDT labeling, pooling the EDT-D_0- and EDT-D_4-labeled proteins, and gel filtration resulted in ~15% sample loss, and subsequent biotinylation, gel filtration, tryptic digestion, and avidin affinity purification resulted in a loss of ~25%. Since these loses occur during sample-handling steps, they can be circumvented by using more automated preparative techniques, hence reducing the amount of protein required per PhIAT analysis. Nevertheless, the data presented in Figure 4 suggest that the efficiency of the PhIAT method is sufficient for most proteome applications.

Assessing the β-Elimination/Michael Addition Labeling of Phosphoproteins. The chemistry of β-elimination of phosphate moieties from pSer and pThr residues in proteins and subsequent Michael addition of various nucleophiles has been well documented[17–19] and has been used with various detection methods to elucidate sites of O-phosphorylation. Phosphoseryl residues were converted into the corresponding cysteic acids, *S*-methylcysteines, alanines, and β-methylaminoalanines to access the sites of phosphorylation in highly phosphorylated peptides and proteins via Edman sequencing.[18] It was found that the *S*-methylcysteines were the best derivatives (using methanethiol as the labeling species) due to the enhanced detection of the corresponding phenylthiohydantoin derivative. In more recent studies, ethanethiol (CH_3CH_2SH, EtSH) was used as the nucleophile to produce *S*-ethylcysteinyl and β-methyl-*S*-ethylcysteinyl residues in highly phosphorylated proteins that were analyzed by LC–MS.[21] This labeling method was extended to using light (CH_3CH_2SH) and heavy (CD_3CD_2SH) isotopic versions on a model phosphoprotein α_{S1}-casein.[14] However, none of the methods discussed has the ability to enrich for phosphopeptides once they have been chemically modified.

Due to the symmetry of the molecule, EDT was selected as the isotopic linker in the PhIAT labeling approach. Attack at the unsaturated β-carbon of either nucleophilic sulfhydryl results in the formation of the same EDT derivative: one sulfur involved in a thioether bond and the other remaining as a free sulfhydryl. This conversion of a pSer or pThr into a residue containing a reactive sulfhydryl makes it amenable to selective biotinlyation using iodoacetyl-PEO-biotin. Conducting the biotinlyation reaction at pH 8.2 promotes only biotinylation of the thiolate without any competitive reactions with amine-containing side chains. However,

(21) Jaffe, H.; Veeranna; Pant, H. C. *Biochemistry* **1998**, *37*, 16211–24.

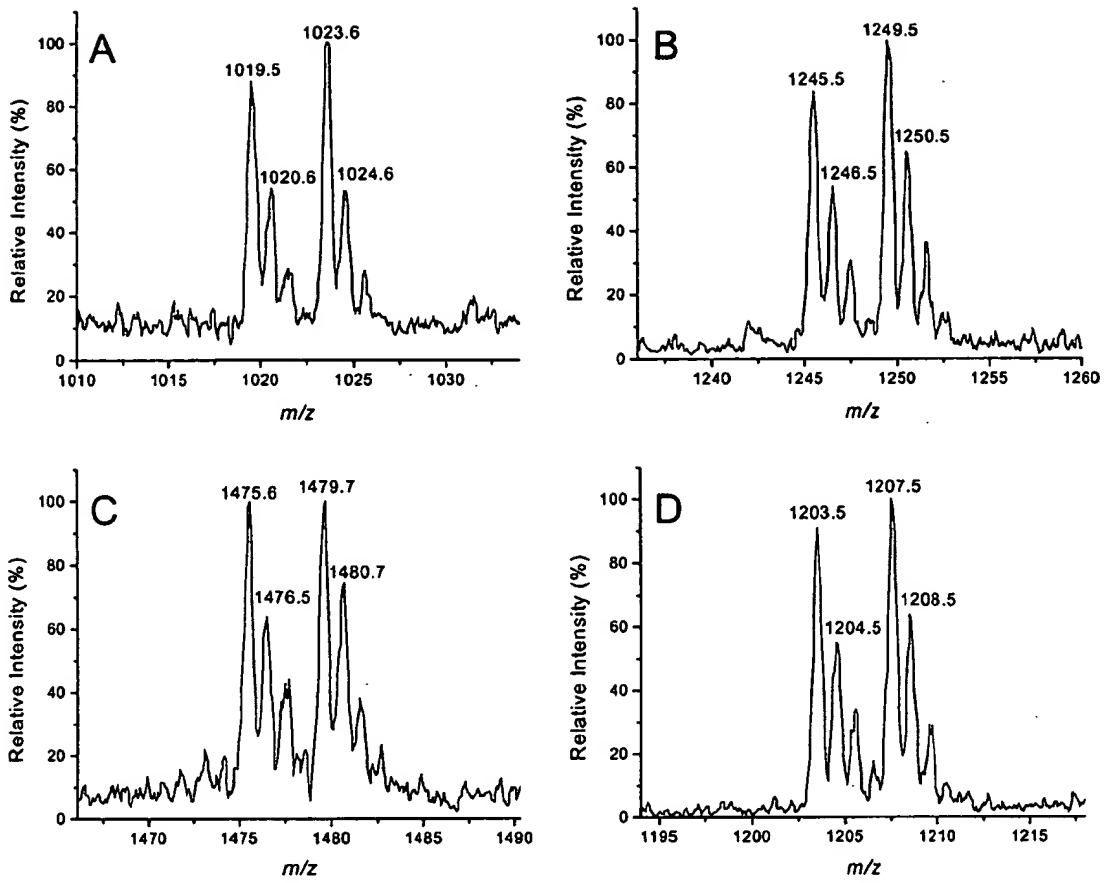

Figure 4. Mass spectra of PhIAT-D_0/D_4-labeled yeast peptides. The enriched mixture of PhIAT-labeled peptides from *S. cerevisiae* was analyzed by capillary reversed-phase liquid chromatography coupled directly on-line to a Finnigan MAT LCQ ion trap mass spectrometer using a 1.0 μg/μL overall sample concentration per 10-μL injection. Each $[M + H]^+$ ion pair features the characteristics of the PhIAT labeling approach: the 4 Da mass difference between ion pairs and the 1:1 ratio indicative of the stoichiometric conversion of identical yeast phosphoproteins to PhIAT-D_0- or PhIAT-D_4-labeled peptides. The yeast protein extract (1 mg) was PhIAT labeled accordingly to Figure 2 using carboxymethylation to block the cysteinyl residues.

to eliminate the biotinylation and any inter- and intramolecular cross-linking that may occur during the β-elimination/addition reaction, the cysteinyl residues must be chemically blocked before PhIAT labeling can be performed (Figure 2). This can be accomplished using either reductive alkylation or performic acid oxidation, both of which have their advantages and disadvantages. Presently, the merits of these two methods are being examined in our PhIAT labeling of phosphoproteomes.

Assessing the PhIAT Labeling of Phosphoproteomes. Due to the preponderance of posttranslational modifications of proteins, there are several other important factors to consider when attempting to PhIAT label phosphoproteins from complex proteomes. O-Linked glycoproteins and lipoproteins may also be susceptible to β-elimination under the reaction conditions used to PhIAT label O-phosphorylation sites. To prevent these nonphosphorylated residues from being PhIAT labeled, the O-linked carbohydrates and lipids can be removed enzymatically using a combination of *O*-glycanse and lipases, respectively, prior to the β-elimination of O-linked phosphates. Conversely, if the O-phosphorylation sites are removed by phosphatases, the sites of O-linked carbohydrates or lipids could be determined using the PhIAT approach. This may be particularly useful when studying mixtures of secreted or membrane-associated proteins that are known to contain a number of O-linked carbohydrates and lipids.

Another important consideration is that prior to cell lysis it would be necessary to add a cocktail of kinase and phosphatase inhibitors to prevent adventitious phosphorylation and dephosphorylation, respectively. This step in proteome sample preparation will be critical when the PhIAT method is used to quantify the differences in the phosphoproteome between a control and a perturbed cell culture or tissue sample.

Due to the highly alkaline conditions used for the β-elimination of the phosphate groups, base hydrolysis of proteins can occur. However, the reaction conditions employed minimizes this unwanted reaction.[19] Future LC–MS/MS analyses will provide a better method to identify nontryptic peptides and thus provide the data to more accurately assess the propensity for base hydrolysis of proteins that may occur during the β-elimination step.

Synthesis of the PhIAT Reagent. As presented in Figure 1, the PhIAT labeling of phosphoproteins has been performed using two separate labeling steps. The first step includes the β-elimination of the phosphate group followed by the addition of EDT, while the second step results in the biotinylation of the EDT-modified peptide. To simplify the overall procedure, it would be advantageous to create a single molecule containing the isotopically labeled EDT as well as the linker domain and biotin tag of

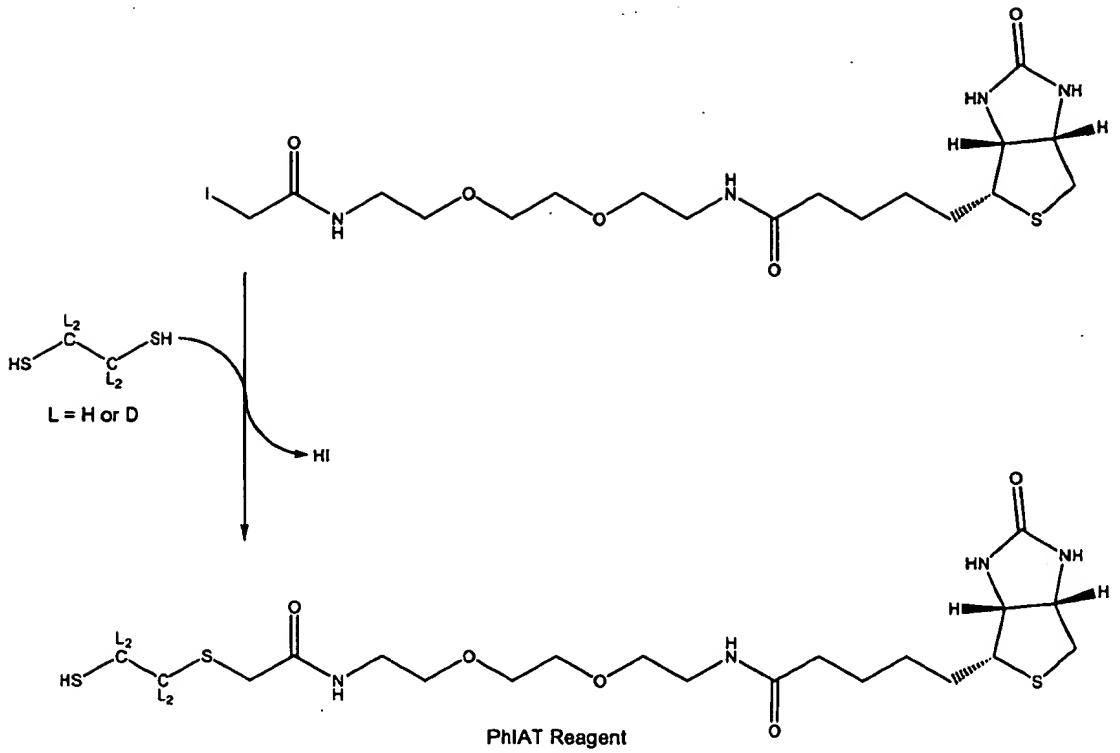

Figure 5. Synthesis of the PhIAT reagent. The isotopically labeled 1,2-ethanedithiol molecule, either $HSCH_2CH_2SH$ (EDT-D_0) or $HSCD_2CD_2SH$ (EDT-D_4), is attached to acetyl-PEO-biotin to create the PhIAT-D_0 or PhIAT-D_4 reagent, respectively. The reaction details are described in the Experimental Section.

iodoacetyl-PEO-biotin, thus permitting the modification of the phosphopeptides to occur in only one step. The reaction scheme for this single PhIAT molecule is shown in Figure 5. Not only will this single PhIAT molecule simplify the procedure but it will also allow for the design of reagents that are isotopically enriched within the linker region of iodoacetyl-PEO-biotin. Importantly, isotopically labeling this linker region can provide a mass difference greater than 4 Da between the light and heavy isotopic forms of the PhIAT reagent. As described in the Experimental Section, the initial efforts demonstrate the efficacy of synthesizing a PhIAT reagent that incorporates all of the critical components of the EDT and iodoacetyl-PEO-biotin molecules.

The mass spectrum of the synthesized PhIAT reagent is shown in Figure 6. Two distinct species are present in the spectrum: the $[M1 + H]^+$ ion at m/z 509.17 and the $[M2 + 2H]^{2+}$ ion at m/z 508.16, corresponding to the monoprotonated PhIAT reagent (RSH) and doubly protonated PhIAT disulfide dimer (RS−SR), respectively. Additional evidence for the presence of the PhIAT disulfide dimer is provided by the signal detected for the $[M2 + H]^+$ ion at m/z 1016.15. The detection of a less intense signal than that observed for the PhIAT disulfide dimer at m/z 601.40 corresponds to the PhIAT molecule disulfide linked to an EDT molecule. These data strongly suggest that the formation of the PhIAT disulfide dimer occurs postreaction. The absence of the $[M + H]^+$ ion signal for iodoacetyl-PEO-biotin (m/z 543.45) or HO-acetyl-PEO-biotin (m/z 433.55) indicates that the 5 molar excess of EDT over iodoacetyl-PEO-biotin is sufficient for synthesis and does not promote substantial EDT polymerization via disulfide bond formation. The yield of purified PhIAT reagent can be increased by reducing the PhIAT disulfide dimer prior to reversed-phase HPLC purification. The demonstrated ability to synthesize the PhIAT reagent represents the first step toward the use of a single reagent to derivatize O-phosphorylated proteins.

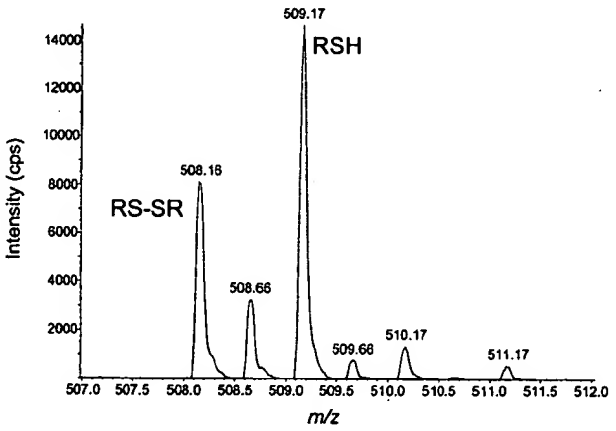

Figure 6. ESI-MS spectrum of the PhIAT reagent. The spectrum shown was obtained by direct infusion of 0.01 $\mu g/\mu L$ of the PhIAT reagent (the PhIAT-D_0 version) into the electrospray interface of a PE Sciex API QStar Pulsar hybrid quadrupole-TOF mass spectrometer using water/methanol (1:1, v/v) with 1% acetic acid as the solvent. The major species at m/z 509.17 is the $[M1 + H]^+$ ion of the monoprotonated PhIAT reagent (RSH) and the minor species at m/z 508.16 is the $[M2 + 2H]^{2+}$ ion of the PhIAT disulfide dimer (RS−SR).

CONCLUSIONS

The PhIAT labeling approach has several important advantages over those using EtSH labeling for the study of global phospho-

rylation states of a proteome. Although the EtSH labeling has proved useful in elucidating the sites of phosphorylation for samples containing one protein, it provides no avenue to enrich the mixture for phosphopeptides, making the analysis of complex samples difficult. Although a preconcentration step involving immobilized metal affinity chromatography (IMAC) may be used to enrich the phosphoprotein/peptide pool prior to EtSH derivatization, the excess Fe^{3+} ions that coelute with the peptides may need to be removed prior to the β-elimination/EtSH addition reaction because of the potential for these ions to facilitate the formation ethane disulfide dimers ($CH_3CH_2S-SCH_2CH_3$).[22] More problematic, however, is the nonspecific binding of nonphosphorylated proteins/peptides that occurs with IMAC. This nonspecificity generally results in substantial contamination of the phosphoprotein/peptide pool and makes any subsequent LC–MS analysis unnecessarily complex. The PhIAT methodology distinguishes itself by modifying the derivatized phosphopeptides with a biotinylated reagent. This modification allows for the enrichment of only those residues that have been isotopically labeled and tagged with biotin by exploiting the high affinity of the biotin–avidin interaction. The resulting reduction in the complexity of the mixture and the removal of high-abundance nonphosphorylated peptides produce a highly enriched sample that will aid in the detection and identification of phosphopeptides.

(22) Netto, L. E.; Stadtman, E. R. *Arch. Biochem. Biophys.* **1996**, *333*, 233–42.

ACKNOWLEDGMENT

We thank the United States Department of Energy Office of Biological and Environmental Research and the National Cancer Institute (Grant CA 86340) for support of portions of this research. Pacific Northwest National Laboratory is operated by Battelle Memorial Institute for the U.S. Department of Energy under contract DE-AC06–76RLO 1830.

Received for review January 18, 2001. Accepted March 30, 2001.

AC010081X